FORM PTO-1390 (REV. 1-98) U S DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER	U.S. APPLICATION NO. (If known, see 37 CFR 1.5)
933-162P	09/646043

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/FI99/00192	March 15, 1999	March 13, 1998

TITLE OF INVENTION

METHOD FOR DETECTING MICROBES FROM AN ENRICHMENT CULTURE

APPLICANT(S) FOR DO/EO/US

HAKALEHTO, Eino E.

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39 (1).
4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date
5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   - a. ☒ is transmitted herewith (required only if not transmitted by the International Bureau). WO 99/47931
   - b. ☒ has been transmitted by the International Bureau.
   - c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☒ A translation of the International Application into English (35 U.S.C. 371(c)(3)).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(2)).
   - a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
   - b. ☐ have been transmitted by the International Bureau.
   - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   - d. ☒ have not been made and will not be made.
8. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. ☒ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern document(s) or information included:

11. ☒ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.-1449 and International Search Report (PCT/ISA/210)

12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.

13. ☒ A FIRST preliminary amendment.
    ☐ A SECOND or SUBSEQUENT preliminary amendment.

14. ☐ A substitute specification.

15. ☐ A change of power of attorney and/or address letter.

16. ☒ Other items or information:
    - 1.) International Preliminary Examination Report (PCT/IPEA/409)
    - 2.) Two (2) sheets of Formal Drawings

U.S. APPLICATION NO (if known, see 37 CFR 1 5)	INTERNATIONAL APPLICATION NO	532 Rec'd PCT/PTO 13 SEP 2000 ATTORNEY'S DOCKET NUMBER
09/646043 NEW	PCT/FI99/00192	933-162P

17. ☒ The following fees are submitted:	CALCULATIONS	PTO USE ONLY
BASIC NATIONAL FEE (37 CFR 1.492(a)(1)-(5): Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO. **$970.00**		
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO **$840.00**		
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO. **$690.00**		
International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) **$670.00**		
International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4). **$96.00**		
ENTER APPROPRIATE BASIC FEE AMOUNT =	$ 970.00	
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☒ 30 months from the earliest claimed priority date (37 CFR 1.492(e)).	$ 130.00	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total Claims	14 - 20 =	0	X **$18.00**	$ 0	
Independent Claims	1 - 3 =	0	X **$78.00**	$ 0	
MULTIPLE DEPENDENT CLAIM(S) (if applicable)	Yes		+ **$260.00**	$ 260.00	
TOTAL OF ABOVE CALCULATIONS =				$ 1360.00	
Reduction of ½ for filing by small entity, if applicable. Verified Small Entity statement must also be filed (Note 37 CFR 1.9, 1.27, 1.28).				$ 0	
SUBTOTAL =				$ 1360.00	
Processing fee of **$130.00** for furnishing the English translation later than ☐20 ☐30 months from the earliest claimed priority date (37 CFR 1.492(f)). +				$ 0	
TOTAL NATIONAL FEE =				$ 1360.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00** per property +				$ 0	
TOTAL FEES ENCLOSED =				$ 1360.00	
				Amount to be: refunded	$
				charged	$

a. ☒ A check in the amount of $ **1360.00** to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account. No. _____ in the amount of $_____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 02-2448.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

Send all correspondence to:
Birch, Stewart, Kolasch & Birch, LLP or Customer No. 2292
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SIGNATURE _____

MURPHY, GERALD M., JR.
NAME

#28,977 (GMM)
REGISTRATION NUMBER

/cqc September 13, 2000

IN THE U.S. PATENT AND TRADEMARK OFFICE

Applicant: HALALEHTO, Eino E.

Int'l. Appl. No.: PCT/FI99/00192

Appl. No.: New Group:

Filed: September 13, 2000 Examiner:

For: METHOD FOR DETECTING MICROBES FROM AN ENRICHMENT CULTURE

PRELIMINARY AMENDMENT

BOX PATENT APPLICATION
Assistant Commissioner for Patents
Washington, DC 20231

September 13, 2000

Sir:

The following Preliminary Amendments and Remarks are respectfully submitted in connection with the above-identified application.

AMENDMENTS

IN THE SPECIFICATION:

Please amend the specification as follows:

Before line 1, insert --This application is the national phase under 35 U.S.C. § 371 of PCT International Application No. PCT/FI99/00192 which has an International filing date of March 15, 1999, which designated the United States of America.--

IN THE CLAIMS:

Please amend the claims as follows:

Claim 5: Line 1, change "any of the claims 1-4" to --claim 1--

Claim 7: Line 1, change "any of the claims 1-6" to --claim 1--

Claim 8: Line 1, change "any of the claims 1-7" to --claim 1--

Claim 11: Line 1, change "any of the claims 1-10" to --claim 1--

Claim 13: Line 1, change "any of the claims 1-12" to --claim 1--

REMARKS

The specification has been amended to provide a cross-reference to the previously filed International Application. The claims have also been amended to delete improper multiple dependents and to place the application into better form for examination. Entry of the present amendment and favorable action on the above-identified application are respectfully requested.

If necessary, the Commissioner is hereby authorized in this, concurrent, and future replies, to charge payment or credit any overpayment to Deposit Account No. 02-2448 for any additional fees required under 37 C.F.R. § 1.16 or under 37 C.F.R. § 1.17; particularly, extension of time fees.

Respectfully submitted,

BIRCH, STEWART, KOLASCH & BIRCH, LLP

By _____
Gerald M. Murphy, Jr., #28,977

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GMM/cqc
933-162P

(Rev. 04/19/2000)

Appln. No 09/646,043

SMALL ENTITY DECLARATION

APPLICANT OR PATENTEE HAKALEHTO, Eino Elias ATTORNEY'S
SERIAL NO. PCT/FI99/00192 ☐ PATENT NO. _____ DOCKET No. 933-162P
FILED OR ISSUED
FOR METHOD FOR DETECTING MICROBES FROM AN ENRICHMENT CULTURE

I(~~we~~) hereby declare that I(~~we~~) am(~~are~~) entitled to the benefit of small entity status with respect to the above-identified application or patent for purposes of paying reduced fees under 35 USC 41(a) & (b) to the U.S. Patent and Trademark Office.

☒ A. INDEPENDENT INVENTOR
I(~~we~~) qualify as a(n) independent inventor(~~s~~) as defined in 37 CFR 1.9(c).

☐ B. INDIVIDUAL NON-INVENTOR
I would qualify as an independent inventor as defined in 37 CFR 1.9(c) if I had made the invention.

☐ SMALL BUSINESS CONCERN
I am ☐ THE OWNER ☐ AN OFFICIAL of the small business concern identified below and am empowered to act on behalf of the concern. The concern qualifies under 37 CFR 1.9(d) and 13 CFR 121.3-18. Rights under contract or law have been conveyed to and remain with the concern and are exclusive unless a checkmark is placed here ☐ and another Declaration on behalf of another entity is filed herewith.

☐ NON-PROFIT ORGANIZATION
I am an official empowered to act on behalf of the non-profit organization identified below. The organization qualifies under 37 CFR 1.9(e), sub-section: ☐ (1) ☐ (2) ☐ (3) ☐ (4). Rights under contract or law have been conveyed to and remain with the organization and are exclusive unless a checkmark is placed here ☐ and another Declaration on behalf of another entity is filed herewith.

I(~~we~~) acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28(b)).

I(~~we~~) hereby declare that all statements made herein of my(~~our~~) own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

A. INDEPENDENT INVENTOR(S) B. INDIVIDUAL NON-INVENTOR(S)

HAKALEHTO, Eino Elias _____ 21.09.2000
Name of Inventor Signature Date

_____ _____ _____
Name of Inventor Signature Date

_____ _____ _____
Name of Inventor Signature Date

C. BUSINESS CONCERN D. NON-PROFIT ORGANIZATION

_____ _____
Name of Concern or Organization Address

By _____ _____
Name of Person Signing Signature

_____ _____
Title Date

METHOD FOR DETECTING MICROBES FROM AN ENRICHMENT CULTURE

Introduction

Salmonella is nowadays one of the most important bacterial contaminants found in food products. Rapid adaptation capability, which is its typical feature, causes difficulties in detecting the bacterium. At present there are over 2000 characterized *Salmonella* strains of which about 100 are clinically and hygienically important. *Salmonella* is a common cause of enteric diseases for both humans and animals. The number of most important strains found during epidemics is about ten. The occurance of *Salmonella* in food often causes large amounts of people to get exposed to an infection. Finding the original source of contamination is a challenging task. Contaminated food or water provide a typical way for the contagion to spread. *Salmonella* belongs to the so called enteric bacteria. Most of the strains cause gastroenteritis.

Normally *Salmonellas* live outside the body of people or host animals in very poor growth conditions. They have to survive and retain their viability e.g. in water, where usually several other microbes compete with them for nutrients. Usually in these circumstances the cells probably develop into some form of resting cells. Once they reach good growth conditions they have to adapt quickly to the new conditions to be able to colonize e.g. the surface epithelial cells of the gastrointestinal tract.

Outside the body *Salmonella* and other enteric bacteria are usually under strong environmental stress. When the *Salmonella* or other micro-organisms get into the body with food, they soon enter the low-pH environment of the stomach, which destroys a large amount of living microbial cells. On the other hand, a low pH also dissociates appendiges on the cell surfaces, of which especially the fimbrias or corresponding structures are used for attaching onto the epithelium. Then after entering the duodenum the *Salmonellas* and other pathogenic enteric species in order to invade the body have to synthesize rapidly the building parts of the different attachment filaments and correspondingly build these appendiges on the cell surfaces. These filaments can be utilised as the basis of immunological methods for detecting microbes, because they are normally strongly immunogenic. They can also be dissociated into single molecules which are their building parts. In the same manner, we can make good use of the detection of the flagellas

that many bacteria synthesize to enable them to move and the flagellin protein, the building parts of the flagellum, in the immunological detection of microbes.

Normally, before using an immunological detection method, the *Salmonella* culture or sample or the culture of enteric bacteria, other bacteria or other micro-organisms must be selectively grown and the desired microbes enriched.

Normally in an enrichment cultivation the sample or culture is usually grown long enough for the number of cells to manifoldly outgrow that of the original sample, usually for at least 12 hours. It is generally supposed that also the amount of antigens increase in about direct ratio to the amount of cells or cell mass.

One of the key goals of food, water and other environmental hygiene is to prevent *Salmonella* bacteria from spreading. For this reason, the detection of *Salmonella* and other similar microbes is an important and broadening field of research and economic activity. The problem in using the traditional bacterial cultivation methods for diagnostics of the *Salmonella* bacteria and other pathogens is the long time needed to attain the results. This causes great expenses e.g. in food industry, where the products often need to be stored up while waiting for the results of the hygiene control or withdrawn from the market or distribution, if the results show contamination with *Salmonella* or other bacteria. Research and development work directed to microbial determinations has recently concentrated on finding more rapid detection methods for microbes.

For clinical sampling in hospitals and for the hygiene mapping of antibiotic resistant microbial strains, more rapid, more reliable and more effective methods are required. These methods should at the same time be useful also for detecting microbes in as simple conditions as possible, even outside laboratories.

Food industry also needs new rapid detection methods and fast methods of enrichment to maintain product safety, shorten storage time and control raw materials. Likewise, different water and environmental analysis, the significance of which has recently grown, need these methods urgently.

Background of the Invention

When detecting microbes from e.g. clinical samples, food samples or environmental samples the microbial concentrations in the original sample are usually so low that so called enrichment methods are needed. These methods increase the amount and concentration of the detected mibrobes in the sample. Microbe specific cultivation methods, which usually involve the use of a selective factor to prevent the multiplication of other microbes, are used. This factor can be a chemical substance, an antibiotic or an equivalent or some physical factor such as the partial pressure of a gas. The pH can also be a selective factor. Often the synergy of the different selective factors can be used in the enrichment culture of the desired microbe in a selection.

The need of having to use enrichment methods in detecting microbes means a loss of time and therefore the shortening of the time used for these procedures is most desirable.

The specific microbial identification often uses antibodies that are produced in animals or cell cultures (immunological methods). They are often used to detect microbes from enrichment cultures, for example. In such a case, the problem may be that the user of the test does not know for sure, if the culture in concern or other sample and the cells in it have enough antigenic molecules for the detection.

The invention can be applied in large scale for monitoring *Salmonella* in foodstuffs. For example in hygiene controls in the meat industry *Salmonella* often exists in so low percentages that the direct detecting using the immunological analysis is with present methods impossible. In these cases an enrichment culture in liquid medium for at least 24 hours is often needed. The enrichment process often divides into two phases: the pre-enrichment stage and the actual enrichment stage. By controlling the conditions of the pre-enrichment growth of the possible *Salmonella* bacteria and the expression of its desired antigens in the sample can be speeded up. Adjusting the conditions of the cultivation also helps to exclude the possibility of cross-reacting, disturbing strains.The enrichment culture is used in the same way as a help to detect and identify many other bacteria and also micro-organisms.

Because the invention makes the process of detecting *Salmonella* and other microbes faster, it can well serve e.g. meat industry and clinical diagnostics, which are in need of rapid methods. In industry the delivery of the products is often put into practice before the microbiological monitoring results are complete. This can lead into major losses if contamination should occur. The rapid methods would make possible to remove the spoiled products early enough.

Description of the Invention.

When growing bacteria species of the genus *Salmonella* and other enteric bacteria on selective nutrient media we noticed that they produced lots of specific antigenic molecules already before the actual microbial population growth, based on the number of cells, was at its maximum. In fact, the concentration of antigen that the specific antibody could detect on the cell surfaces had substantially lowered from its record figures as the cell growth in the enrichment culture grew near its maximum. For this reason the use of an immunological detection method can take place earlier than in the presently known methods, directly following the so called stationary phase before the number of cells, defined with e.g. the calculation methods based on the colony count, has markedly increased. For example, the temperature, the composition of the nutrient medium, antibodies or other selective molecules and the control of partial pressures of various gases can be used as selective factors in the enrichment culture.

According to the method of this invention, the identification process of microbes from various samples can be made more rapid by making use of the changes in the expressions of the different surface structures of the microbes according to the changes in the growth phase and growth conditions. By exploiting the "enhanced" enrichment of antigens we could e.g. observe the increased concentrations of type 1 fimbrial antigens in 3 – 10 hours from the onset of the cultivation (Examples 4 – 5). These fimbrias or their building parts, which the microbes produce e.g. to enable them to attach to the epithelial cells of the alimentary tract, can be made use of in methods for detecting microbes more rapidly than in the methods according to the present knowledge.

In one possible form of application, after a suitable period of enrichment it is possible to detect the microbes by taking advantage of filter extraction (Finnish patent No. 93742). The

immunological detection itself can be carried out with an immunostrip, the ELISA-method, a luminometric method or other corresponding apparatus or method using antibodies, which are suitable specifically for extracted surface structures. This procedure may, in theory, add the sensitivity of the detection when the amount of cells is still relatively small.

In theory, the attachment characteristics could be made use of also in coating the plunger of the injection syringe used in taking the sample (US. Patent No. 5,846,209). The plunger used in taking the sample could then be changed into a plunger coated with specific antibodies against to the attachment molecules or molecules that imitate the object of the attachment molecules of the searched bacteria. When handling fluid samples the plunger need not be changed. Once the microbes are brought into suitable growth conditions they begin to express their attachment molecules and attach with them to the molecules on the surface of the plunger. The attachment is verified with some suitable method (e.g. electrically or optically).

Examples 4 – 5 describe the reactions of a peptide antibody, produced against different *Salmonella* strains, with bacterial cultures in different growth phases. On the basis of the results from these experiments we were able to demonstrate high immunological reaction levels already before the beginning of the actual logarithmic growth phase. When producing antibody to the fimbrial proteins and to peptides derived from them, we noticed strong immunological reactivities already in 3 – 5 hours with antibodies produced against fimbrial peptides (Examples 4 – 5). The results implicate that the *Salmonella* type 1 fimbrial sequence, used in the experiment as source material in the production of synthetic peptides, would express outstandingly strongly already before the actual logarithmic growth phase or immediately at its beginning.

When examining the growth of the bacterial cultures on different media and in different growth conditions, we noticed that e.g. the growth of a *S. enteritidis*-culture in a selective medium (RVS) was at its strongest after 3 – 6 hours the temperature being +37°C and the mass of the cultivation grew close to its maximum in 8 hours (Example 4).

The basis of the phenomenon described above is the theory that reaching favourable conditions in the intestines of man or a warm-blooded animal the food-borne *Salmonella* bacterium or other enteric bacterium firstly produces fimbrias and other molecular structures required for attachment, in order to be able to stick to this favourable environment, where nutrients are

readily available. It is also possible that already in the stationary phase cells the attachment proteins or their precursors are at least partially prepared in the cytoplasm or in a corresponding site from where they can be mobilized in the fastest way from the point of view of the bacterial cell.

Example 1: Cultivation of *Salmonella* on RVS medium

Salmonella enteriditis strain 9,12:-g, m:-, phage type 4 (IHS 59813) and *Salmonella typhimurium* strain 4,5,12:i:1,2, phage type 1 (IHS 59929) were kept at 37 °C in THG medium (5% tryptone, 2.5% yeast extract and 1% glucose) and seeded in the intervals of two weeks during the experiment. The *Salmonella* strains were obtained from the National Public Health Institute (Helsinki, Finland). Cultivation was started from 3 – 4 days old culture by inoculating 5% of the culture to a fresh RVS medium (Rappaport-vassiliadis soya peptone broth, Oxoid, England), which is selective for *Salmonella*. The RVS suspension was cultivated in shaked Erlenmeyer flasks (each 100 ml) at two different temperatures: +37 °C and +43 °C. Samples were taken every hour.

Example 2: Description of the peptide

The sequence in the peptide synthesis was traced from the *Salmonella typhimurium* type 1 fimbriae. In order to select a specific sequence differing from the corresponding *E.coli* type 1 fimbriae, the two sequences were compared with each other. The sequence of 18 amino acids Ala Ser Phe Thr Ala Ile Gly Asp Thr Thr Ala Gln Val Pro Phe Ser Ile Val was selected. The peptide was synthetized as molecules, in which 4 identical peptides were joined together from one end forming thus a multiple-antigen peptide (MAP). The peptides were synthetized with Millipore PerSeptive 9050 Plus automated peptide synthetizer and with Fmoc synthesis strategy. Fmoc-Lys(Fmoc)-OH was used as the backbone for the branched structure.

Example 3: Immunizations

Peptides were used for immunization without any conjugation to carrier molecules. Rabbits were immunized with 500 µg of MAP-peptide injected subcataneously. The immunization solution contained also Freund's complete adjuvant. The boosters were injected in intervals of two weeks.

The solution contained also Freund's incomplete adjuvant. Together with the immunizations the rabbits were bleeded in order to take serum samples, which were stored in the freezer (-20 °C).

Example 4:

For studying the reactions of the anti-peptide antibody against bacterial cultures in different growth phases, a "growth speed-ELISA" experiment was carried out using two bacterial cultures differing from each other in cell density. *Salmonella enteriditis* strain IHS 59813 was used in the experiment, the growth medium was RVS broth and the temperature +37 °C. Plate cultures were started simultaneously with ELISA meassurements. The bacterial densities were in the beginning of the experiment 1.3 E+6 and 1.0 E+4. Results from the ELISA experiment and the plate counts are presented in Table 1 and in Figure 1. "Growth speed ELISA" was carried out as follows:

1. The microtiter plates were treated with glutaraldehyde:
   a) 150 µg of 0,5% glutaraldehyde was added to the wells and
   b) the microplate was incubated at room temperature in a shaker for 15 minutes.
2. Washes: The glutaraldehyde was poured from the wells and the wells were washed twice with 200 µl of 1 x ELISA washing solution (5mM Tris + 0.15 M NaCl + 0.05 Tween20).
3. The antigen was added to the desired wells as an Assay Buffer dilution (1:1) 50 µl per well (Assay Buffer PBS + 1%BSA + 0.05%NaCL + 1mM EDTA).
   Into the zero wells, where no antigen was added, 50 µl 1 x washing solution was pipetted per well. Thenafter, the microplate was wrapped in a folio and incubated overnight in a cold room.
4. On the following day the wells were poured empty and washed three times as described above.
5. For blocking 1.5% BSA/TBS solution was added to the wells (200µl/well) and the microplate was incubated on a shaker for one hour.
6. The wells were washed as in 4.
7. The primary antibodies were added (peptide serum H463):
   a) Serum dilution (1:100) was pipetted (50 µl per well).
   b) The plate was incubated on a shaker for 0.5 hours at room temperature.
8. The wells were washed four times as above.

9. The secondary antibodies were added:

a) Secondary antibody (anti-rabbit) dilution 1:1000 was pipetted 50 µl per well.

b) The plate was incubated on a shaker for 0.5 hours at room temperature.

10. The plates were washed three times as above and once with Afos Buffer (200 µl/well).

11. Colour solution (pNPP) was pipetted (50 µl/well) to the plate.

12. Absorbance was measured with an ELISA-reader (wave length 405 nm) after 15 minutes, half an hour, one and a half hours and three hours after the addition of colour.

Table 1. The absorbance figures for IHS 59813 antigen (one hour after the addition of colour) and bacterial densities as the function of time.

Bacterial density in the beginning $1.3*10^6$

Time	0 h	2.5 h	3.5 h	4.5 h	5.5 h	6.5 h	7.5 h	8.5 h	9.5 h
0-absorbance	0.245	0.245	0.245	0.232	0.227	0.227	0.227	0.230	0.234
Absorbance	0.228	0.233	0.248	0.464	0.531	0.483	0.387	0.345	0.376
Bacterial density	$1.3*10^6$	$9.7*10^5$		$1.8*10^6$		$7.7*10^8$		$1.3*10^9$	$1.8*10^9$

Bacterial density in the beginning $1.0*10^4$

Time	0 h	2.5 h	3.5 h	4.5 h	5.5 h	6.5 h	7.5 h	8.5 h	9.5 h
0-absorbance	0.222	0.226	0.227	O.227	0.227	O.227	0.225	0.225	0.225
Absorbance	0.216	0.238	0.235	0.229	0.218	0.249	0.346	0.332	0.314
Bacterial density	$1.0*10^4$	$1.1*10^4$		$1.9*10^4$		$5.3*10^8$		$1.8*10^8$	$2.7*10^{10}$

Example 5:

For studying the reactions of the anti-peptide antibody against bacterial cultures in different growth phases, a "growth speed-ELISA" experiment was carried out using two different *Salmonella* strains (IHS 59813 ans IHS 59929). The growth medium was RVS broth and the cultivation temperature 43 °C . Plate cultures were started simultaneously with the ELISA experiment. The experiment was carried out as described in the Example 4. The results are presented in the Table 2 and Figure 2.

Table 2. The absorbance figures for IHS 59813 antigen and IHS 59929 antigen (three hours after the addition of colour) and bacterial densities as the function of time.

Salmonella strain IHS 59813

Time	0 h	1 h	2 h	3 h	4 h	5 h	6 h	7 h	8 h
0-absorbance	0.346	0.346	0.346	0.336	0.323	0.323	0.323	0.357	0.355
Absorbance	0.369	0.362	0.428	0.739	1.202	1.120	0.928	0.826	0.859
Bacterial density	$1.0*10^7$		$1.3*10^8$		$1.4*10^9$		$1.5*10^8$		$1.7*10^8$

Salmonella strain IHS 59929

Time	0 h	1 h	2 h	3 h	4 h	5 h	6 h	7 h	8 h
0-absorbance	0.360	0.350	0.344	0.344	0.344	0.367	0.355	0.355	0.355
Absorbance	0.392	0.427	0.480	0.733	1.136	0.867	0.697	0.726	0.671
Bacterial density	$7.7*10^6$		$1.9*10^7$		$4.0*10^7$		$2.2*10^8$		$5.5*10^8$

Literature

Hakalehto, E. Finnish patend n:o 93742, Menetelmä ja laite solujen osoittamiseksi, (A method and an apparatus for detecting cells), 1995.

Hakalehto, E. US. Patent n:o 5,846,209, Syringe comprising an adhering substrate for microbes, 1998.

Amended Claims

1. A microbiological determination method, characterized in, that the bacteria are detected from their cultivation medium clearly prior to the peak of the population growth using the antigens which the cells express soon after their inoculation to the enrichment medium, before the actual growth phase or in the beginning of it.

2. A method according to the claim 1, characterized in, that the microbial antigens are detected immunologically using antibodies directly after the stationary phase.

3. A method according to the claim 2, characterized in, that the microbial antigens are detected immunologically in 3–4.5 hours after the onset of the enrichment culture.

4. A method according to any of the claims 2–3, characterized in, that the detected antigens are proteins.

5. A method according to any of the claims 1–4, characterized in, that the detected antigens are fimbrial proteins.

6. A method according to the claim 5, characterized in, that the detected antigens are type 1 fimbrial proteins or comparable to them.

7. A method according to any of the claims 1–6, characterized in, that the microbial antigens are detected with antibodies, which have been produced against the synthetic peptide Ala Ser Phe Thr Ala Ile Gly Asp Thr Thr Ala Gln Val Pro Phe Ser Ile Val or a derivative thereof.

8. A method according to any of the claims 1–7, characterized in, that the detected microbes are enteric bacteria.

9. A method according to the claim 8, c h a r a c t e r i z e d in, that the detected microbes are fecal coliforms.

10. A method according to the claim 9, c h a r a c t e r i z e d in, that the detected microbes belong to genus *Salmonella*.

11. A method according to any of the claims 1–10, c h a r a c t e r i z e d in, that the microbes are incubated prior to the immunological detection in their optimal growth temperature.

12. A method according to the claim 11, c h a r a c t e r i z e d in, that the microbes are incubated prior to the detection at temperatures about 37 °C.

13. A method according to any of the claims 1–12, c h a r a c t e r i z e d in, that the microbes are incubated prior to the detection at temperatures above 42 °C.

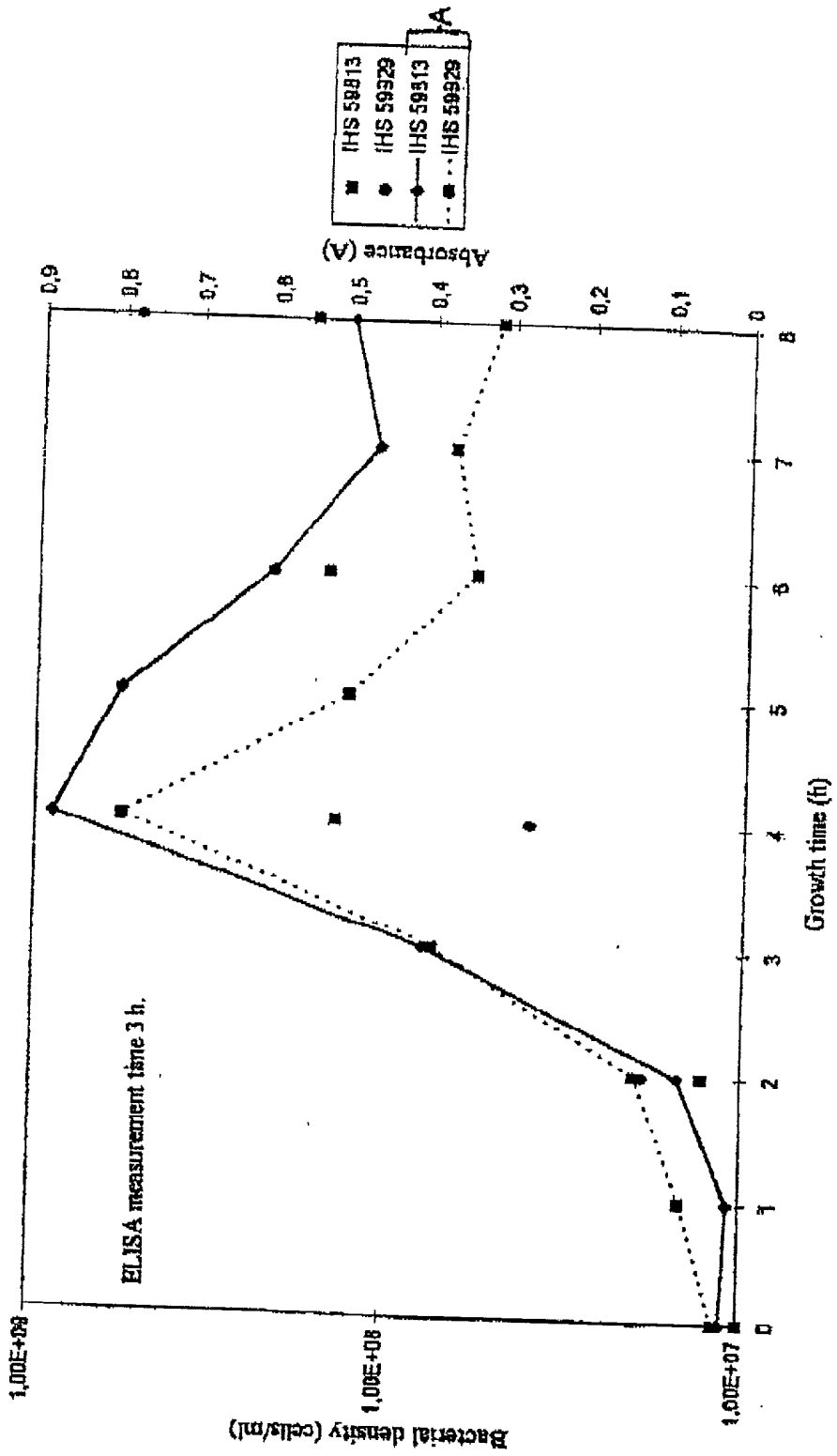

Figure 2. "Growth speed ELISA" 2.12.1997 and growth as a function of time 1.12.1997: strains IHS 59813 and IHS 59929 at +43 °C. Primary serum in ELISA H463 1:100.

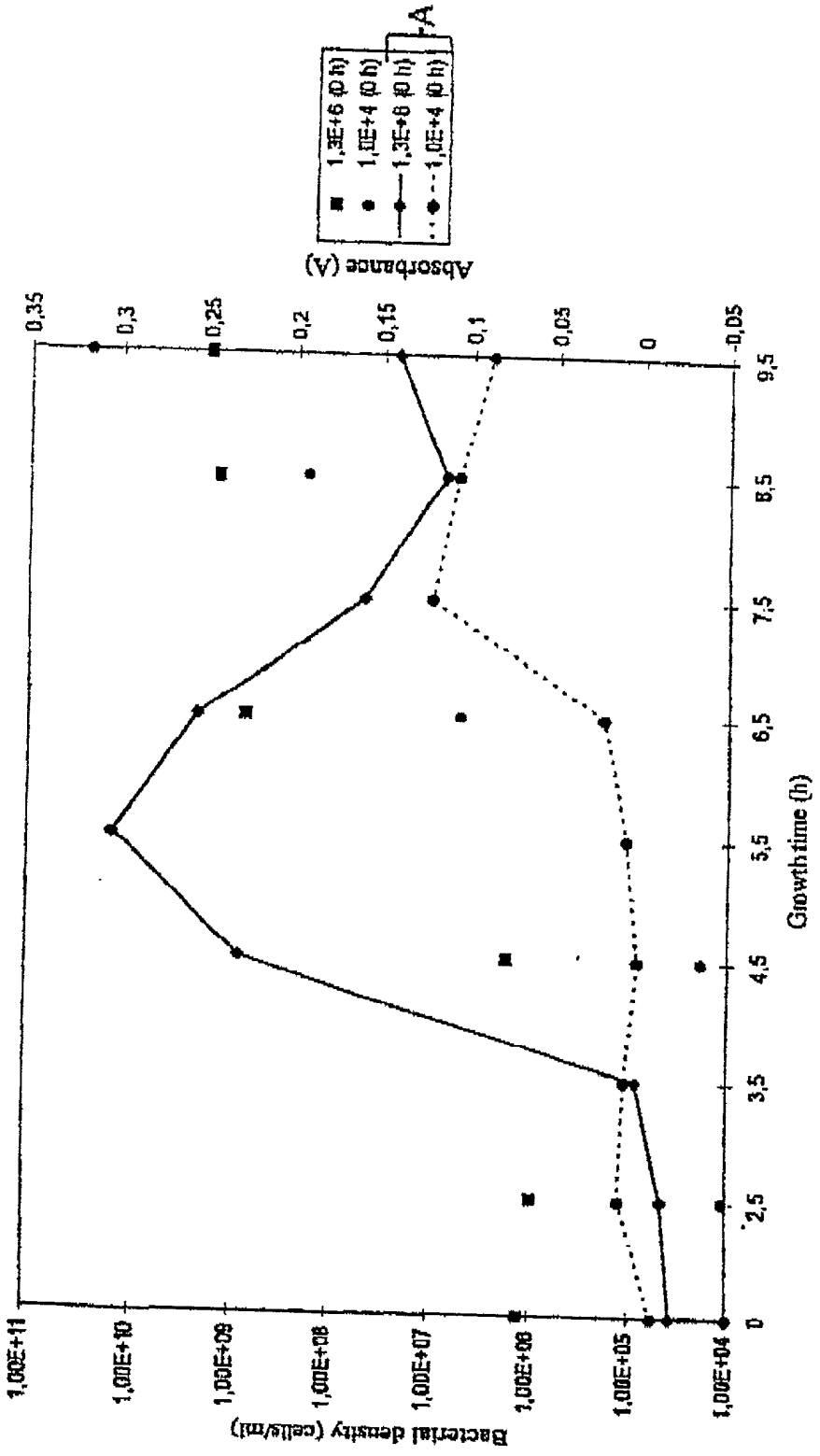

Figure 1. "Growth speed ELISA" 14.11.1997: UHS 59813 at +37 °C, bacterial density was in the beginning 1.3E+6 and 1.0E+4 cells/ml and bacterial density of the strain as a function of time.

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ATTORNEY DOCKET NO.
933-162P

PLEASE NOTE: YOU MUST COMPLETE THE FOLLOWING:

COMBINED DECLARATION AND POWER OF ATTORNEY FOR PATENT AND DESIGN APPLICATIONS

As a below named inventor, I hereby declare that: my residence, post office address and citizenship are as stated next to my name; that I verily believe that I am the original, first and sole inventor (if only one inventor is named below) or an original, first and joint inventor (if plural inventors are named below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

Insert Title: → METHOD FOR DETECTING MICROBES FROM AN ENRICHMENT CULTURE

the specification of which is attached hereto. If not attached hereto,

Fill in Appropriate Information — For Use Without Specification Attached: →

the specification was filed on _____ as
United States Application Number _____ ;
and amended on _____ (if applicable); and/or
the specification was filed on March 15, 1999 as PCT
International Application Number PCT/FI99/00192 ; and was
amended under PCT Article 19 on _____ (if applicable)

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, §1.56.

I do not know and do not believe the same was ever known or used in the United States of America before my or our invention thereof, or patented or described in any printed publication in any country before my or our invention thereof or more than one year prior to this application, that the same was not in public use or on sale in the United States of America more than one year prior to this application, that the invention has not been patented or made the subject of an inventor's certificate issued before the date of this application in any country foreign to the United States of America on an application filed by me or my legal representatives or assigns more than twelve months (six months for designs) prior to this application, and that no application for patent or inventor's certificate on this invention has been filed in any country foreign to the United States of America prior to this application by me or my legal representatives or assigns, except as follows.

I hereby claim foreign priority benefits under Title 35, United States Code, §119 (a)-(d) of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

Insert Priority Information: (if appropriate) →

Prior Foreign Application(s)			Priority Claimed	
980571 (Number)	Finland (Country)	3/13/1998 (Month / Day / Year Filed)	☒ Yes	☐ No
(Number)	(Country)	(Month / Day / Year Filed)	☐ Yes	☐ No
(Number)	(Country)	(Month / Day / Year Filed)	☐ Yes	☐ No
(Number)	(Country)	(Month / Day / Year Filed)	☐ Yes	☐ No

I hereby claim the benefit under Title 35, United States Code, §119(e) of any United States provisional application(s) listed below.

Insert Provisional Application(s): (if any) →

(Application Number)	(Filing Date)
(Application Number)	(Filing Date)

All Foreign Applications, if any, for any Patent or Inventor's Certificate Filed More than 12 Months (6 Months for Designs) Prior to the Filing Date of This Application:

Insert Requested Information: (if appropriate) →

Country	Application Number	Date of Filing (Month / Day / Year)

I hereby claim the benefit under Title 35, United States Code, §120 of any United States and/or PCT application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States and/or PCT application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, §1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application:

Insert Prior U.S. Application(s): (if any) →

(Application Number)	(Filing Date)	(Status — patented, pending, abandoned)
(Application Number)	(Filing Date)	(Status — patented, pending, abandoned)

I hereby appoint the following attorneys to prosecute this application and/or an international application based on this application and to transact all business in the Patent and Trademark Office connected therewith and in connection with the resulting patent based on instructions received from the entity who first sent the application papers to the attorneys identified below, unless the inventor(s) or assignee provides said attorneys with a written notice to the contrary:

16 —

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Full Name of First or Sole Inventor: Insert Name of Inventor → Insert Date This Document is Signed → Insert Residence → Insert Citizenship → Insert Post Office Address →

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Residence (City, State & Country):

CITIZENSHIP:

POST OFFICE ADDRESS (Complete Street Address including City, State & Country):

* DATE OF SIGNATURE